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(54) Title: CYCLIC PEPTIDE VACCINES FOR TREATMENT AND PREVENTION OF DIABETES (57) Abstract The present invention provides immunogenic oligopeptides derived from the Major Histocompatibility Complex (MHC) glycoprotein protein sequences for use in compositions and methods for the treatment, prevention and diagnosis of diabetes.		

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CYCLIC PEPTIDE VACCINES FOR TREATMENT AND PREVENTION OF DIABETES

CROSS REFERENCE TO RELATED APPLICATIONS

This is a continuation in part of USSN 60/014,790, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to novel compositions and methods for inhibiting immune responses associated with autoimmune diseases. In particular, it relates to vaccination with peptides from hypervariable regions of MHC molecules associated with insulin dependent diabetes mellitus (IDDM).

IDDM is the result of selective destruction of the insulin-producing β cells in the pancreas (*see, e.g., Eisenbarth, N. Engl. J. Med.* 314:1360-1368 (1986) and Reich, *et al., Diabetes Reviews* 1:293 (1993)). Insulin regulates the cellular uptake and metabolism of glucose, and its deficiency leads to hyperglycemia diabetic acidosis, and diabetic coma. The disease usually appears in individuals before the age of 20 and affects about 0.5% of the Caucasian population worldwide.

Current therapeutic approaches aimed at prevention of IDDM are based on non-antigen-specific inhibition of T cells (*e.g.*, cyclosporin A) or by providing β cell rest (*e.g.*, parenteral administration of insulin). In patients who already have diabetes, transplantation of the pancreas or insulin producing β cells is also being explored.

These prior art methods fail to provide a simple self-mediated method for specifically eliminating immune responses restricted by glycoproteins encoded by MHC alleles associated with the autoimmune response associated with IDDM. Because autoreactive T cells are responsible for the anti- β cell attack which leads to diabetes, therapeutic strategies which intervene at the stage of TCR engagement would be particularly useful. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods of inducing an immune response against an HLA molecule associated with IDDM, such as HLA-DQ molecules. The methods comprise administering to a patient an immunologically effective amount of a pharmaceutical composition comprising an adjuvant and a cyclic immunogenic MHC polypeptide from a hypervariable region of a HLA-DQ molecule, typically, the β chain of the HLA-DQ molecule. The peptides include from about 10 to about 50 residues, but typically consist of about 20 to about 30 residues. A preferred peptide comprises amino acid residues 57-78 of a protein encoded by DQB1*0302. A particularly preferred peptide is CAAEYWNSQKEVLERTRAELDTVC.

The peptides may be administered according to standard protocols, typically by parenteral administration. An adjuvant such as alum may be included. The compositions of the invention may be administered prophylactically or therapeutically. For instance, the compositions can be administered to pre-diabetics to prevent or ameliorate the onset of symptoms.

Definitions

The term "peptide" is used interchangeably with "oligopeptide" or "polypeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the α -amino and carbonyl groups of adjacent amino acids.

The term "cyclic peptide" refers to peptides in which the N-terminal residue is linked to the C-terminal residue either directly or through an intermediate. Examples of links between the two residues include disulfide bonds and thioether linkages as described below.

An "immunogenic MHC polypeptide" of the present invention is a polypeptide capable of eliciting an immune response against an MHC molecule associated with a deleterious immune response in a patient, such as IDDM. As set forth in more detail below, the sequence of residues in the polypeptide will be identical to or substantially identical to a polypeptide sequence in the MHC molecule. Thus, a polypeptide of the invention that has a sequence "from a hypervariable region of an HLA-DQ molecule" is polypeptide that has a sequence either identical to or substantially

identical to the naturally occurring MHC amino acid sequence of the region.

As used herein a "hypervariable region" of an MHC molecule is a region of the molecule in which polypeptides encoded by different alleles at the same locus have high sequence variability or polymorphism. The polymorphism is typically concentrated in the $\alpha 1$ and $\alpha 2$ domains of Class I molecules and in the $\alpha 1$ and $\beta 1$ domains of Class II molecules. The number of alleles and degree of polymorphism among alleles may vary at different loci. For instance, in HLA-DR molecules all the polymorphism is attributed to the β chain and the α chain is relatively invariant. For HLA-DQ, both the α and β chains are polymorphic. For instance, a hypervariable region spanning residues 57 through 78 of the DQ β chain can be used. For a description of hypervariable regions in various HLA gene products *see, Fundamental Immunology, supra*.

As used herein, the term "adjuvant" refers to any substance which, when administered with or before an antigen, increases and/or qualitatively affects the immune response against the antigen in terms of antibody formation and/or the cell-mediated response. Exemplary adjuvants for use in the present invention are provided below.

The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the MHC polypeptides of this invention do not contain materials normally associated with their *in situ* environment, *e.g.*, other surface proteins on antigen presenting cells. Even where a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated polypeptides of this invention do not contain such endogenous co-purified protein.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in a oligopeptide by an amide bond or amide bond mimetic.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence of the third hypervariable region of various human and murine Class II β chains.

Figure 2 shows the percent diabetes in NOD mice treated with various doses of cyclic peptides of the invention.

Figure 3 shows the results of ELISA analysis of serum derived from mice

treated with peptides of the invention.

Figure 4 shows the results of ELISA analysis of serum derived from mice treated with peptides of the invention.

Figure 5 shows the results of ELISA analysis of serum derived from mice treated with peptides of the invention using different boosting regimens.

Figure 6 shows the results of analysis of T cell reactivity in mice treated with peptides of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides immunogenic polypeptides derived from MHC glycoprotein protein sequences for use in compositions and methods for the treatment, prevention and diagnosis of IDDM. The polypeptides are capable of inducing an immune response against glycoproteins encoded by MHC alleles associated with the disease. In preferred embodiments the polypeptides of the invention are cyclized and derived from hypervariable regions of the α or β chain of an HLA-DQ molecule.

The glycoproteins encoded by the MHC have been extensively studied in both the human and murine systems. Many of the histocompatibility proteins have been isolated and characterized. For a general review of MHC glycoprotein structure and function, see *Fundamental Immunology, supra*.

MHC molecules are heterodimeric glycoproteins expressed on cells of higher vertebrates and play a role in immune responses. In humans, these molecules are referred to as human leukocyte antigens (HLA). MHC glycoproteins are divided into two groups, class I and class II, which differ structurally and functionally from each other. In general, the major function of MHC molecules is to bind antigenic peptides and display them on the surface of cells.

Human MHC Class II molecules are encoded by genes in the HLA-DR, -DP, and -DQ subregions. These glycoproteins comprise two chains, the α and β chains, which extend from the membrane bilayer. Each subunit in MHC Class II molecules consist of globular domains, referred to as $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$. All except the $\alpha 1$ domain are stabilized by intra-chain disulfide bonds typical of molecules in the immunoglobulin superfamily. The N-terminal portions of the α and β chains, the $\alpha 1$ and $\beta 1$ domains, contain hypervariable regions which are thought to comprise the majority of the antigen-

binding sites (*see*, Brown *et al.*, *Nature* 364:33-39 (1993)).

The HLA-DQ subregion contains two genes encoding α chains (DQA1 and DQA2) and three genes encoding β chains (DQB1, DQB2, and DQB3). The DQA1 and DQB1 genes encode the HLA-DQ molecule, while the other genes are pseudogenes.

5 As noted above, each MHC allele encodes proteins which comprise hypervariable regions and antigen binding sites specific for particular sets of antigenic peptides. If the peptides bound by the MHC molecule are from an autoantigen, allergen or other protein associated with a deleterious immune response, the hypervariable region of the MHC molecule can be used to produce immunogenic polypeptides which will elicit an
10 immune response against the MHC molecule. These polypeptides are therefore useful in targeting particular gene products associated with deleterious immune responses because the immune response against the MHC molecule will inhibit antigen presentation associated with the deleterious immune response (*see*, PCT application No. US93/12351).

Thus, immunization with the polypeptides will be haplotype specific and
15 result only in the inhibition of the immune response mediated by the target molecules, while leaving other alleles unaffected. Most individuals are heterozygous at each MHC locus. Therefore, haplotype specific therapy of disease by immunization with polypeptides of the disease susceptibility gene products of MHC genes offers a novel means of immunotherapy.

20 For the treatment of humans, peptides from the hypervariable regions of HLA molecules associated with IDDM are used. A number of HLA alleles are associated with IDDM. These include DR4, DR3, and DQ. For a discussion of the association of various HLA alleles with IDDM, *see*, Green, *Current Topics in Microbiology and Immunology* 164:3 (1990), Todd, *Current Topics in Microbiology and Immunology* 164:17
25 (1990), Erlich, *Current Topics in Microbiology and Immunology* 164:41 (1990), Michelsen *et al.*, *Current Topics in Microbiology and Immunology* 164:57 (1990), and Lo, *Current Topics in Microbiology and Immunology* 164:71 (1990). The peptides of the invention are preferably from the hypervariable region of an HLA-DQB1 molecule.

In the example below, a peptide consisting of residues 56-77 (in which the histidine at residue 56 is replaced by cysteine) of the murine MHC class II I-A^{g7} β sequence is used as the immunogen. This synthetic peptide has the following sequence:
I-A^{g7} β 56-57 C56

5 C-S-A-E-Y-Y-N-K-Q-Y-L-E-R-T-R A-E-L-D-T-A-C-NH₂

DQB1 is the human equivalent of the murine I-A^{g7} β chain gene. A comparison of the sequences the third hypervariable region of human and murine Class II β chains is shown in Figure 1. A preferred peptide is one from the third hypervariable region of DQB1*0302. An exemplary peptide comprises the following sequence.

10 A-A-E-Y-W-N-S-Q-K-E-V-L-E-R-T-R A-E-L-D-T-V

In preferred embodiments, the peptides of the invention are cyclized. Methods for cyclizing peptides are described in detail below. In those cases in which the peptides are cyclized by disulfide linkages, one of skill will recognize that the peptides will further comprise cysteine residues either within the peptide or at each terminus.

Polypeptides suitable for use in the present invention can be obtained in a variety of ways. Conveniently, they can be synthesized by conventional techniques employing automatic synthesizers, such as the Beckman, Applied Biosystems, or other commonly available peptide synthesizers using well known protocols. They can also be synthesized manually using techniques well known in the art. See, e.g. Stewart and Young, *Solid Phase Peptide Synthesis*, (Rockford, Ill., Pierce), 2d Ed. (1984).

Alternatively, DNA sequences which encode the particular MHC polypeptide may be cloned and expressed to provide the peptide. Cells comprising a variety of MHC genes are readily available, for instance, they may be obtained from the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," 6th edition (1988) Rockville, Maryland, U.S.A. The National Institute of General Medical Sciences Catalog of Cell Lines (NIGMS) Human Genetic Mutant Cell Repository, Camden, NJ; and ASHI Repository, Bingham and Women's Hospital, 75 Francis Street, Boston, MA 02115 are also useful sources. Cell lines expressing appropriate DQ alleles include GMO6824A (DQB1*0201) and GMO6821A (DQB1*0302), both of which are available from NIGMS.

Standard techniques can be used to screen cDNA libraries to identify sequences encoding the desired sequences (see, Sambrook et al., *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Fusion proteins (those consisting of all or part of the amino acid sequences of two or more proteins) can be recombinantly produced. In addition, using *in vitro* mutagenesis techniques, unrelated proteins can be mutated to comprise the appropriate sequences.

MHC glycoproteins from a variety of natural sources are also conveniently isolated using standard protein purification techniques. Peptides can be purified by any of a variety of known techniques, including, for example, reverse phase high-performance liquid chromatography (HPLC), ion-exchange or immunoaffinity chromatography, separation by size, or electrophoresis (See, generally, Scopes, R., *Protein Purification*, Springer-Verlag, N.Y. (1982)).

It will be understood that the immunogenic MHC polypeptides of the present invention may be modified to provide a variety of desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide. For instance, the peptides can be modified by extending, decreasing the amino acid sequence of the peptide. Substitutions with different amino acids or amino acid mimetics can also be made.

The peptides employed in the subject invention need not be identical to peptides disclosed in the Example section, below, so long as the subject peptides are able to induce an immune response against the desired MHC molecule. Thus, one of skill will recognize that a number of conservative substitutions (described in more detail below) can be made without substantially affecting the activity of the peptide.

Single amino acid substitutions, deletions, or insertions can be used to determine which residues are relatively insensitive to modification. Substitutions are preferably made with small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues. The effect of single amino acid substitutions may also be probed using D-amino acids. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased immunogenicity may also be achieved by such substitutions, compared to the parent peptide. In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to

avoid, for example, steric and charge interference which might disrupt binding.

The substituting amino acids, however, need not be limited to those naturally occurring in proteins, such as L- α -amino acids, or their D-isomers. The peptides may be substituted with a variety of moieties such as amino acid mimetics well known to those of skill in the art.

The individual residues of the immunogenic MHC polypeptides can be incorporated in the peptide by a peptide bond or peptide bond mimetic. A peptide bond mimetic of the invention includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. VII (Weinstein ed., 1983). Several peptide backbone modifications are known, these include, $\psi[\text{CH}_2\text{S}]$, $\psi[\text{CH}_2\text{NH}]$, $\psi[\text{CSNH}_2]$, $\psi[\text{NHCO}]$, $\psi[\text{C}(\text{OCH}_2)]$ and $\psi[(\text{E}) \text{ or } (\text{Z}) \text{CH}=\text{CH}]$. The nomenclature used above, follows that suggested by Spatola, above. In this context, ψ indicates the absence of an amide bond. The structure that replaces the amide group is specified within the brackets.

Amino acid mimetics may also be incorporated in the peptides. An "amino acid mimetic" as used here is a moiety other than a naturally occurring amino acid that conformationally and functionally serves as a substitute for an amino acid in a polypeptide of the present invention. Such a moiety serves as a substitute for an amino acid residue if it does not interfere with the ability of the peptide to illicit an immune response against the appropriate MHC molecule. Amino acid mimetics may include non-protein amino acids, such as β - γ - δ -amino acids, β - γ - δ -imino acids (such as piperidine-4-carboxylic acid) as well as many derivatives of L- α -amino acids. A number of suitable amino acid mimetics are known to the skilled artisan, they include cyclohexylalanine, 3-cyclohexylpropionic acid, L-adamantyl alanine, adamantylacetic acid and the like. Peptide mimetics suitable for peptides of the present invention are discussed by Morgan and Gainor, (1989) *Ann. Repts. Med. Chem.* 24:243-252/

As noted above, the peptides employed in the subject invention need not be identical, but may be substantially identical, to the corresponding sequence of the target MHC molecule. Therefore, the peptides may be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where

such changes might provide for certain advantages in their use. The polypeptides of the invention can be modified in a number of ways so long as they comprise a sequence substantially identical (as defined below) to a sequence in the target region of the MHC molecule.

5 Alignment and comparison of relatively short amino acid sequences (less than about 30 residues) is typically straightforward. Comparison of longer sequences may require more sophisticated methods to achieve optimal alignment of two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482, by
10 the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (USA)* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the
15 best alignment (*i.e.*, resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.*, on a nucleotide-by-nucleotide basis) over a window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned
20 sequences over the window of comparison, determining the number of positions at which the identical residues occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

25 As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical
30 differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and

isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The polypeptides of the invention typically comprise at least about 10 residues and more preferably at least about 15 residues. In certain embodiments the peptides will not exceed about 50 residues and typically will not exceed about 30 residues. For instance, the DQB1*0302 peptides described below consist of 22 residues with out the additional cysteine residues at each end. In other embodiments, the entire subunit (α or β chain) or large portions of the molecules are used. For instance, the polypeptides can comprise an extracellular domain from an MHC subunit (about 90-100 residues). Typically, the N-terminal domain (β 1 or α 1) is used. The entire extracellular region (*e.g.*, β 1 and β 2 or α 1 and α 2 of class II molecules or α 1, α 2 and α 3 of class I molecules) from the subunit can also be used. Thus, a wide range of polypeptide sizes may be used in the present invention.

In the preferred embodiments of the invention, the immunogenic peptides are cyclized. Any method commonly used to produce cyclized oligopeptides can be used to produce the peptides of the invention. For example, in certain embodiments the peptides will include cysteine residues at both termini, which allow the production of cyclic peptides through disulfide linkages. Treatment of a such a peptide with an oxidizing agent such as oxygen, iodine or similar agent will produce a cyclic peptide which may be further purified using chromatographic or other methods of chemical purification. Construction of cyclic peptides can also be accomplished through thioether linkages. For instance, N-bromoacetyl-derivatized peptides can be reacted with sulfhydryl-containing residues, such as cysteine. Cyclization occurs by reaction of the free sulfhydryl of cysteine in the peptide with the bromoacetyl group to form a thioether linkage (Robey *et al.*, *Anal. Biochem.* 177:373-7 (1989) and U.S. Patent No. 5,066,716).

Other methods of constructing cyclic peptides are known to those skilled in

the art. These include side chain-side chain, side chain-main chain and main chain-main chain cyclizations. In addition, linkers can be used to join the amino and carboxyl termini of a peptide. The linker is capable of forming covalent bonds to both the amino and carboxyl terminus. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. The linkers may be joined to the carboxyl and amino terminal amino acids through their side groups (e.g., through a disulfide linkage to cysteine) or through the alpha carbon amino and carboxyl groups of the terminal amino acids.

For a general discussion of suitable methods for cyclization see, Hruby and Bonner in *Methods in Molecular Biology, Volume 35: Peptide Synthesis Protocols* Pennington and Dunn eds (Humana Press, Totowa NJ, 1994). For instance, cyclizations may include formation of carba analogs and thioethers (Lebl *et al.* in *Peptides 1986* Proceedings of the 19th European Peptide Symposium pp. 341-344; Robey *et al.*, *Anal. Biochem.* 177:373-7 (1989) and U.S. Patent No. 5,066,716), bis-thioethers (Mosberg *et al.* *JACS* 107:2986-2987 (1985)), azopeptides (Siemion *et al.* *Mol. Cell. Biochem.* 34: (1991)), and other cyclic structures, such as bridging structures (Charpentier, M., *et al.*, *J. Med. Chem.* 32(6):1184-1190 (1989), Thaisrivongs, S., *et al.*, *J. Med. Chem.* 34(4):127 (1991) and Ozeki, E., *et al.*, *Int. J. Peptide Protein Res.* 34:111 (1989)).

Cyclization from backbone-to-backbone positions may also be used.

Bridging is a special type of cyclization in which distant sites in a peptide are brought together using separate bridging molecules or fragments. Bridging molecules may include, for example, succinic anhydride molecules (Charpentier, B., *et al.*, *supra*), and carboxymethylene fragments (Thaisrivongs, S., *et al.*, *supra*). Bridging by metals can also be used (Ozeki, E., *et al.*, *supra*).

In some embodiments, the peptides include two or more cystine residues. The cystines can be substituted or added within the peptide or at either terminus. The position of the cystines is not critical so long as disulfide linkages can form between them which allow the production of cyclic peptides. For example, treatment of such a peptide with an oxidizing agent such as oxygen, iodine or similar agent will produce a cyclic peptide which may be further purified using chromatographic or other methods of chemical purification.

In addition to use of peptides, antibodies raised against peptides of the invention can be used to inhibit autoimmune responses, such as IDDM. Antibodies can be raised to the peptides of the present invention using techniques well known to those of skill in the art. Anti-idiotypic antibodies can also be generated. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

A number of immunogens can be used to produce antibodies specifically reactive with the peptides. For instance, the entire MHC molecule or fragments containing the desired sequence can be used. Synthetic peptides as disclosed here can be used either in linear form or cyclized.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.*, GST, keyhole limpet hemocyanin, *etc.*), or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (*see, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Description of techniques for preparing such monoclonal antibodies are found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method proceeds by injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or

"hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate (preferably mammalian) host. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about .1 mM, more usually at least about 50 μ M, and most preferably at least about 1 μ M or better.

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g., Huse et al. (1989) Science 246: 1275-1281; and Ward, et al. (1989) Nature 341: 544-546; and Vaughan et al. (1996) Nature Biotechnology, 14: 309-314*).

Frequently, the peptides and antibodies of the invention will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

The antibodies of this invention can also be administered to an organism (*e.g., a human patient*) for therapeutic purposes (*e.g., to inhibit an autoimmune response*). Antibodies administered to an organism other than the species in which they are raised are often immunogenic. Thus, for example, murine antibodies administered to a human often induce an immunologic response against the antibody (*e.g., the human anti-mouse*

antibody (HAMA) response) on multiple administrations. The immunogenic properties of the antibody are reduced by altering portions, or all, of the antibody into characteristically human sequences thereby producing chimeric or human antibodies, respectively.

Chimeric antibodies are immunoglobulin molecules comprising a human and non-human portion. More specifically, the antigen combining region (or variable region) of a humanized chimeric antibody is derived from a non-human source (*e.g.*, murine) and the constant region of the chimeric antibody (which confers biological effector function to the immunoglobulin) is derived from a human source. The chimeric antibody should have the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule. A large number of methods of generating chimeric antibodies are well known to those of skill in the art (*see, e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 5,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369). An alternative approach is the generation of humanized antibodies by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. *See* Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989) and WO 90/07861.

In one preferred embodiment, recombinant DNA vector is used to transfect a cell line that produces an antibody against a peptide of the invention. The novel recombinant DNA vector contains a "replacement gene" to replace all or a portion of the gene encoding the immunoglobulin constant region in the cell line (*e.g.*, a replacement gene may encode all or a portion of a constant region of a human immunoglobulin, or a specific immunoglobulin class), and a "target sequence" which allows for targeted homologous recombination with immunoglobulin sequences within the antibody producing cell.

In another embodiment, a recombinant DNA vector is used to transfect a cell line that produces an antibody having a desired effector function, (*e.g.*, a constant region of a human immunoglobulin) in which case, the replacement gene contained in the recombinant vector may encode all or a portion of a region of an antibody and the target sequence contained in the recombinant vector allows for homologous recombination and targeted gene modification within the antibody producing cell. In either embodiment, when only a portion of the variable or constant region is replaced, the resulting chimeric

antibody may define the same antigen and/or have the same effector function yet be altered or improved so that the chimeric antibody may demonstrate a greater antigen specificity, greater affinity binding constant, increased effector function, or increased secretion and production by the transfected antibody producing cell line, *etc.*

5 In another embodiment, this invention provides for fully human antibodies. Human antibodies consist entirely of characteristically human polypeptide sequences. The human antibodies of this invention can be produced in using a wide variety of methods (*see, e.g.,* Larrick *et al.*, U.S. Pat. No. 5,001,065). In one preferred embodiment, the human antibodies of the present invention are produced initially in trioma cells. Genes
10 encoding the antibodies are then cloned and expressed in other cells, particularly, nonhuman mammalian cells. The general approach for producing human antibodies by trioma technology has been described by Ostberg *et al.* (1983), *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.*, U.S. Pat. No. 4,634,666. The antibody-producing cell lines obtained by this method are called triomas because they are
15 descended from three cells; two human and one mouse. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

Model Systems for In vivo Testing

20 An animal model for spontaneous IDDM and thyroiditis has been developed in the BB rat (*see, e.g.,* Rossini *et al. Autoimmunity* 8:221-235 (1991)). As in humans, the rat disease is controlled in part by the genes encoding the MHC antigens, is characterized by islet infiltration, and is associated with the presence of anti-islet antibodies. The I-E equivalent Class II MHC antigens appear to be involved in manifestation of the autoimmune diseases in the BB rat. Biotard, et al., *Proc. Natl. Acad. Sci. USA* 82:6627 (1985).
25

In another spontaneous model, the NOD mouse strain (H-2^k) is a murine model for autoimmune IDDM. The disease in these animals is characterized by anti-islet cell antibodies, severe insulitis, and evidence for autoimmune destruction of the β -cells. Kanazawa, et al., *Diabetologia* (1984) 27:113. The disease can be passively transferred
30 with lymphocytes and prevented by treatment with cyclosporin-A (Ikehara, et al., *Proc. Natl. Acad. Sci. USA* (1985) 82:7743; Mori, et al.), *Diabetologia* (1986) 29:244. Untreated animals develop profound glucose intolerance and ketosis and succumb within

weeks of the onset of the disease. Seventy to ninety percent of female and 20-30% of male animals develop diabetes within the first six months of life. Breeding studies have defined at least two genetic loci responsible for disease susceptibility, one of which maps to the MHC. Characterization of NOD Class II antigens at both the serologic and molecular level suggest that the susceptibility to autoimmune disease is linked to I-A β . Baxter *et al.* *Autoimmunity* 9:61-67 (1991) and Kikutani *et al.* *Adv. Immunol.* 51:285 (1992).

Formulation and Administration

The peptides or antibodies (typically monoclonal antibodies) of the present invention and pharmaceutical compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent deleterious immune responses. Suitable formulations are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985).

The immunogenic peptides or antibodies of the invention are administered prophylactically or to an individual already suffering from the disease. In particular, the composition of the invention can be administered to pre-diabetics or early stage diabetics. In addition, first degree relatives of IDDM patients can now be screened for autoantibodies to three common islet cell antigens, GAD, ICS and IA-2. Those testing positive in these or other diagnostic assays can be treated with the compositions of the present invention. In this way the onset of the disease symptoms can be prevented or ameliorated.

The peptide compositions are administered to a patient in an amount sufficient to elicit an effective immune response to the MHC molecule from which the peptides are derived. An amount adequate to accomplish this is defined as "therapeutically effective dose" or "immunogenically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 0.1 mg to about 1.0 mg per 70 kilogram patient, more commonly from about 0.5 mg to about 0.75 mg per 70 kg of body weight. Boosting dosages are typically from about 0.1 mg to about 0.5 mg of peptide using a boosting regimen over weeks to months depending upon

the patient's response and condition. A suitable protocol would include injection at time 0, 4, 2, 6, 10 and 14 weeks, followed by further booster injections at 24 and 28 weeks.

It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of autoimmune disease. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In some circumstances, loading doses followed by boosting doses may be required. The resulting immune response helps to cure or at least partially arrest symptoms and/or complications. Vaccine compositions containing the peptides are administered prophylactically to a patient susceptible to or otherwise at risk of the disease to elicit an immune response against the target MHC antigen.

The pharmaceutical compositions (containing either peptides or antibodies) are intended for parenteral or oral administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95 % of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25 %-75 %.

As noted above, the peptide compositions are intended to induce an immune response to the peptides. Thus, compositions and methods of administration suitable for maximizing the immune response are preferred. For instance, peptides may be introduced into a host, including humans, linked to a carrier or as a homopolymer or heteropolymer of active peptide units. Alternatively, the a "cocktail" of polypeptides can be used. A mixture of more than one polypeptide has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies to a number of epitopes. For instance, polypeptides comprising sequences from hypervariable regions of α and β chains may be used in combination. Useful carriers are well known in the art, and include, e.g., KLH, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like.

The use of more than one polypeptide is particularly useful to enhance the immune response against polypeptides of the invention. As demonstrated below, although the polypeptides may be derived from self MHC molecules expressed in the patient, they can induce an immune response. In some instances, the immune response to the self polypeptide may not be sufficiently strong. In these instances, it may be necessary to break tolerance to the polypeptide. The compositions may comprise one or more of the foreign polypeptides that are sufficiently similar to the self polypeptides to induce an immune response against both the foreign and self polypeptides (*see*, Mamula *et al.* *J. Immunol.* 149:789-795 (1992)). Suitable proteins include synthetic polypeptides designed for this purpose or polypeptide sequences from homologous proteins from natural sources, such as proteins encoded by a different allele at the same locus as the self polypeptide.

The compositions also include an adjuvant. As used here, number of adjuvants are well known to one skilled in the art. Suitable adjuvants include incomplete Freund's adjuvant, alum, aluminum phosphate, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP),
5 N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween
10 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against the immunogenic peptide.

A particularly useful adjuvant and immunization schedule are described in Kwak et al. *New Eng. J. Med.* 327:1209-1215 (1992). The immunological adjuvant described there comprises 5% (wt/vol) squalene, 2.5% Pluronic L121 polymer and 0.2 %
15 polysorbate in phosphate buffered saline.

The concentration of immunogenic peptides of the invention in the pharmaceutical formulations can vary widely, i.e. from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of
20 administration selected.

The peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into a host, the recombinant vaccinia virus expresses the immunogenic
25 peptide, and thereby elicits an immune response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (*Nature* 351:456-460 (1991)). A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., *Salmonella typhi*
30 vectors and the like, will be apparent to those skilled in the art from the description herein.

The DNA encoding one or more of the peptides of the invention can also be administered to the patient. This approach is described, for instance, in Wolff *et. al.*, *Science* 247: 1465-1468 (1990) as well as U.S. Patent Nos. 5,580,859 and 5,589,466.

In order to enhance serum half-life, the peptides may also be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or other conventional techniques may be employed which provide an extended serum half-life of the peptides. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4, 235,871, 4,501,728 and 4,837,028.

The peptides or antibodies of the invention can also be used for diagnostic purposes. For instance, peptides can be used to screen for autoantibodies to ensure that the vaccination has been effective. Antibodies can be used to detect the presence of particular MHC molecules associated with disease.

The following examples are offered by way of illustration, not by way of limitation.

Example 1

This example describes the use of cyclized peptides from the murine I-A^{E7} β chain (including amino acids 56-77).

The I-A^{E7} β 56-77 C56 peptide sequence is from the third hypervariable region of NOD mouse MHC class II I-A^{E7} with a cysteine replacing the histidine at the position 56. This synthetic peptide has the following sequence:

I-A^{E7} β 56-77 C56

C-S-A-E-Y-Y-N-K-Q-Y-L-E-R-T-R-A-E-L-D-T-A-C-NH₂

Synthesis and characterization of the cyclic peptide

The linear peptide I-Ag7 β 56-77C56 was synthesized by Merrifield solid phase peptide synthesis (SPPS) on an automated peptide synthesizer (433 A Applied Biosystems, Foster City USA) generally as described by Luu *et al.* *Int. J. Peptide Protein Res.* 47:91-96 (1996). The peptide was assembled from carboxy terminal end at 0.25 millimole scale on Rink amide MBHA resin (substitution level 0.55 millimoles per gram).

HOBt/HBTU coupling strategy was used for acylation of amines on the resin and piperidine was used for the deprotection of Fmoc protected α -amine of the amino acid on the resin. The side chain protection strategy is given in Table 1. Our earlier studies indicated coupling problems after the 7th the residue from the C terminus and therefore a double coupling of amino acids was carried out after the seventh residue.

N-methylpyrrolidinone (NMP) was used as solvent for coupling/deprotection reactions and dichloromethane(DCM) was used for final washing of peptide resin. The deprotection was monitored by measuring the conductivity of Fmoc released.

Table 1. Side chain protecting groups used in the peptide synthesis.

Amino Acid	Side Chain Protecting Group
S, T, Y, D, and E	t-Butyl (t-Bu)
R	2,3,5,7,8-Pentamethyl-chloraman-6-sulfonyl (Pmc)
K, and W	Butyloxycarbonyl (Boc)

After successful assembly of the peptide chain on the resin, the peptide resin was dried in vacuum for 2 hours and subjected peptide cleavage protocol. The resin was suspended in 10 ml of trifluoroacetic acid(TFA) containing 0.6 g of 4-methylmercaptophenol and 1 ml of 4-methoxybenzenethiol. The suspension was mixed for 2 hours at room temperature and then filtered into 1000 ml of organic solvent(pentane:acetone 4:1). The precipitate was allowed to settle for 2 hours at room temperature. The clear solvent was removed by decantation and centrifugation. The precipitate was washed with pentane acetone mixture (50 ml each time 5 times) and finally with pentane (50 ml twice). The crude linear peptide was dried in vacuum for 1 hour. It was subjected to reverse phase HPLC using a C18 column.

Cyclization of the crude peptide:

The crude linear peptide (100 mg) was dissolved in 100 ml of 0.5% acetic acid containing 15% acetonitrile. Two grams of commercially available intramolecular disulfide bond forming resin (Ekathiox from Ekagen Corp. San Carlos, CA) was mixed

with it and the suspension was gently tumbled overnight at room temperature. The formation of cyclic peptide was monitored by HPLC and estimation of free SH groups in solution by Ellman Reagent. The resin was removed by filtration and the filtrate was used for isolation of cyclic peptide.

5

The crude cyclic peptide solution obtained above was directly loaded on HPLC with C18 reverse phase column. The cyclic peptide was eluted using acetonitrile water gradient. The pure fractions as indicated by HPLC were pooled and lyophilized and the pure cyclized peptide was characterized by HPLC, Amino acid analysis and mass spectrometry. The peptides were stored at -20°C until they were used.

10

Example 2

This example describes animal studies using the peptides described in Example 1.

15

In vivo studies to demonstrate the ability of the cyclic I-A^{E7} peptide vaccine to protect NOD mice from IDDM were carried out. The entire project was divided into low dose, mid dose, and high dose studies as follows.

Low Dose:

20	<u>Treatment group/mouse</u>	<u>n=</u>
	5μg peptide/150 μl Alum	5
	50μg peptide/ 150 μl Alum	5
	150μ Alum	5
	150μl PBS	5

25

Mid Dose:

	<u>Treatment group/mouse</u>	<u>n=</u>
	50μg peptide/150 μl Alum	5
	100μg peptide/ 150 μl Alum	10
30	200μg peptide/ 150 μl Alum	7
	150μl Alum	10
	150μl PBS	5

High dose:

Treatment group/ mouse

n-

400 μ g peptide/ 150 μ l Alum

600 μ g peptide/ Alum

5

Mice were administered treatment in three subcutaneous dorsal sites. The peptide was emulsified in an Alum adjuvant; which had previously been demonstrated not to cause protection from or delay the onset of diabetes in the NOD mouse (data not shown).

10

For each treatment group, the initial injection was at 5 weeks of age, and was followed by three boosts at intervals of 2-3 weeks, with the final injection taking place at 12 weeks of age. Screening for diabetes was begun at 12 weeks of age, and was performed by urinalysis with Ames Glucose Diastix according to the manufacturer's instructions (Ames, Elkhart, IN).

15

The results of these experiments are shown in Figure 2. There it can be seen that at dose levels of 50 μ g and above, statistically significant protection against diabetes as compared to controls (Alum alone) was observed.

20

Western blot analysis was used to determine the reactivity of antiserum from the mice 24 weeks after immunization with the peptides. These results showed that antiserum from mice receiving 50, 100 and 200 μ g of peptide, was reactive with the IA^{g7} β chain, whereas control serum from untreated NOD mice showed no reactivity.

25

In addition ELISA analysis was performed to test the reactivity of the antiserum from the mice. Plates were coated with a linear peptide from IA^s, which differs from the administered peptide by three residues (*see*, Figure 1), and the administered peptide in linear and cyclized form. Binding of antibodies MKD6 (anti-IA^d), 10.2.16 (anti-IA^{g7.s.k}) and serum from mice treated and control mice was then tested. As can be seen in Figure 3, serum from treated mice contains high titers of antibodies that bind IA^{g7}, but not IA^s. Figure 4 shows the results of a similar experiment, with two additional controls and serum titrated at different concentrations.

30

The effects of boosting regimen and animal age on the ability to induce antibodies specific to I-A^{g7} were also investigated. In these studies, groups of 10 mice at 3, 4, 5, and 6 weeks of age were injected with peptides as described above (initial dose =

50 or 100 μ g/mouse), followed by boost of the indicated dose at 4 or 6 weeks. ELISA analysis as described for Figure 4 was then carried out. As shown in Figure 5, a specific immune response against the I-A^{g7} peptide was boosted by administration of 100 μ g/mouse at 4 weeks or 6 weeks, with slightly better results seen at 6 weeks.

5 Figure 5 also shows the results of experiments designed to examine whether antibodies raised against I-A^{g7} also recognize peptides in which the arginine at position 67 is changed to glutamine in accordance with the sequence of the corresponding region of IA^S (see, Figure 1). As can be seen in Figure 5, this amino acid substitution was not recognized by the antibodies. Thus, the specificity of the immune response for I-A^{g7} is
10 apparently based on changes at other positions in these molecules.

 Figure 6 shows the results of experiments designed to examine T cell mediation of the antibody responses. In these experiments, animals received *in vivo* treatment with either an Alum or PBS control or with 50, 100 or 200 μ g of the peptide of the invention, and were then sacrificed at either 4 or 6 weeks later. Reactivity of both
15 splenic and lymph node-derived T cells to *in vitro* challenge with immunogens and mitogens was determined by measuring uptake of [³H] thymidine according to standard techniques. As can be seen in Figure 6, there was a response to the mitogens ConA and LPS by T cells in 100% of the mice in every treatment, indicating that the peptide of the invention does not cause any general immune suppression. *In vitro* challenge with either
20 the linear or cyclic I-Ag7 peptide induced a splenic response in treated mice; the percentage of mice showing a T cell response increased with the dosage of primary *in vivo* treatment, and control mice initially treated with Alum or PBS did not show this response. Additionally, challenge with peptides other than those of the invention (GAD peptide or mouse serum albumin (MSA)) did not induce T cell proliferation, indicating that T cells
25 specifically reactive with these antigens were not present. T Cells reactive with linear or cyclic peptides of the invention were present in the spleen, but were not detected in the lymph nodes. These results indicate that circulating T cells specifically reactive with the peptides are present in animals which receive peptides of the invention. They also suggest that the antibody response detected above is T cell-mediated.

Example 3

This example described preparation of a cyclized peptides based on sequences from DQB1*0302.

A. Synthesis and Characterization of Cyclic DQB1*0302 Peptide, Cyclized Through Thioether Bond Formation

Peptide sequence: CH₃CONH-AAEYWNSQKEVLERTRAELDTVC-NH₂

|—————S—————|

1. *Synthesis of peptide.*

The linear peptide DQB1*302 was synthesized by Merrifield solid phase synthesis (SPPS) on an automated peptide synthesizer (433A, Applied Biosystems, Foster City, California, USA) as described generally by Luu *et al.*, *Int. J. Peptide Protein Res.* 47:91-96 (1996). The peptide was assembled from carboxy terminal end at 0.25 millimole scale on Rink amide MBHA resin (substitution level 0.49 mmols per grain). HOBt/HBTU coupling strategy was used for acylation of amines on the resin and piperidine was used for the deprotection of Fmoc protected α -amine of the amino acid on the resin. The side chain protection strategy is given in Table 2. N-methylpyrrolidinone (NMP) was used as a solvent for coupling/deprotection reactions and dichloromethane (DCM) was used for final washing of peptide resin. The deprotection was monitored by measuring the conductivity of Fmoc released.

Table 2. Side chain protection groups used in peptide synthesis.

Amino acid	Side chain protection group
Asn(N), Cys(C), His(H), Gln(Q)	Trt
Arg(R)	Pbf
Lys(K)	Boc
Ser(S), Thr(T), Tyr(Y), Asp(D), Glu(E)	t-Bu
Ala(A) and Leu(L)	no protection group

After successful assembly of peptide chain on the resin, the peptide resin was dried in vacuum for 2 hours and subjected to bromoacetylation procedure. The resin was suspended in DMF containing 0.1% (by volume) DIEA and bromoacetic anhydride in 4:1 molar excess was added. The suspension was mixed for 2 hours at room temperature. A small amount of slurry was taken and washed with DMF, methanol 3 times and finally with DCM. Then ninhydrine test was performed which was negative (no blue or purple color). It indicated that the reaction was complete. The resin was then washed as described above and dried under vacuum.

After bromoacetylation, the peptide resin was dried in vacuum for two hours and subjected to peptide cleavage protocol. The resin was suspended in 10 ml of trifluoroacetic acid (TFA), 0.75 g of 4-(Methylmercapto)phenol and 0.75 ml of 4-Methoxybenzenethiol. The suspension was mixed for 2 hours at room temperature and then filtered into 1000 ml of pentane-acetone solution (4:1 by volume). The clear solvent was removed by centrifugation. The precipitate (peptide) was washed with pentane-acetone solution three more times (50 ml each time) and finally with pentane (50 ml). The crude linear peptide was dried in vacuum for 1 hour. It was subjected to reverse phase analytical HPLC using C18 column for quality control.

2. Cyclization of peptide by forming thioether bridge.

For cyclization, the linear peptide was dissolved in argon sparged aqueous 0.1M sodium bicarbonate solution at concentration 1mg/ml. Cyclization reaction was allowed to go overnight. The solution was centrifuged and the supernatant was checked by HPLC. The retention time for cyclic peptide was about 4 minutes less than for linear peptide. Peptide solution was lyophilized and purified.

3. Peptide purification.

Peptide was purified on preparative reverse phase HPLC system applying gradient method. The solvents used were: 0.1% TFA in water and 0.1% TFA in acetonitrile. The fractions were checked on analytical HPLC. The pure fractions as indicated by HPLC were pooled and lyophilized. Overall purity of peptide was over 78%.

4. Peptide characterization.

Peptide was characterized by the chromatogram from analytical HPLC and by mass-spectrometry. Molecular weight of cyclic peptide was 2564 amu.

5 B. Synthesis and Characterization of Cyclic DQB1*0302 Peptide, Cyclized Through Disulfide Bond Formation.

Peptide sequence: CAAEYWNSQKEVLERTRAE LDTVC-NH₂.



10 1. Synthesis of peptide.

The linear peptide DQB1*302 was synthesized by Merrifield solid phase synthesis (SPPS) on an automated peptide synthesizer (433A, Applied Biosystems, Foster City, California, USA) as described generally by Luu *et al.*, *Int. J. Peptide Protein Res.* 47:91-96 (1996). The peptide was assembled from carboxy terminal end at 0.25 millimole scale on Rink amide MBHA resin (substitution level 0.49 mmol per gram).
 15 HOBt/HBTU coupling strategy was used for acylation of amines on the resin and piperidine was used for the deprotection of Fmoc protected α -amine of the amino acid on the resin. The side chain protection group is given in Table 3. N-methylpyrrolidinone (NMP) was used as a solvent for coupling/deprotection reactions and dichloromethane
 20 (DCM) was used for final washing of peptide resin. The deprotection was monitored by measuring the conductivity of Fmoc released.

Table 3. Side chain protection groups used in peptide synthesis.

25	Amino acid	Side chain protection group
	Asn(N), Cys(C), His(H), Gln(Q)	Trt
	Arg(R)	Pbf
	Lys(K)	Boc
30	Ser(S), Thr(T), Tyr(Y), Asp(D), Glu(E)	t-Bu
	Ala(A) and Leu(L)	no protection group

After successful assembly of peptide chain on the resin, the peptide resin was dried in vacuum for 2 hours and subjected to peptide cleavage protocol. The resin was suspended in 10 ml of trifluoroacetic acid (TFA), 0.75g of 4-(Methyl-mercapto)phenol and 0.75 ml of 4-Methoxy-benzenethiol. The suspension was mixed for 2 hours at room temperature and then filtered into 1000 ml of pentane-acetone solution (4:1 by volume). The clear solvent was removed by centrifugation. The precipitate (peptide) was washed with pentane-acetone solution three more times (50 ml each time) and finally with pentane (50 ml). The crude linear peptide was dried in vacuum for 1 hour. It was subjected to reverse phase analytical HPLC using C18 column for quality control.

2. *Cyclization of peptide through disulfide bond formation.*

For cyclization peptide was dissolved in water at concentration 1mg/ml, pH of the solution was brought to 8-9 by adding ammonium hydroxide. This solution was kept at 40°C for 3-4 hours so that peptide cyclized. After lyophilization peptide was checked by the same analytical HPLC. The retention time for cyclic peptide is about 4 minutes less than for linear peptide.

3. *Peptide purification.*

Peptide was purified on preparative reverse phase HPLC system applying gradient method. The solvents used were: 0.1 % TFA in water and 0.1 % TFA in acetonitrile. The fractions were checked on analytical HPLC. The pure fractions as indicated by HPLC were pooled and lyophilized. Overall purity of peptide was over 78%.

4. *Peptide characterization.*

Peptide was characterized by the chromatogram from analytical HPLC and by mass-spectrometry. Molecular weight of cyclic peptide was 2625 amu.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of inducing an immune response against an HLA-DQ molecule in a patient, the method comprising administering to the patient an immunologically effective amount of a pharmaceutical composition comprising an adjuvant and a cyclic immunogenic MHC polypeptide from a hypervariable region of a HLA-DQ molecule.
2. The method of claim 1, wherein the hypervariable region is in the β chain of the HLA-DQ molecule.
3. The method of claim 1, wherein the cyclic immunogenic MHC polypeptide consists of about 20 to about 30 residues.
4. The method of claim 1, wherein the immunogenic MHC polypeptide comprises amino acid residues 57-78 of a protein encoded by DQB1*0302.
5. The method of claim 1, wherein the immunogenic MHC polypeptide has the amino acid sequence CAAEYWNSQKEVLERTRAELDTVC.
6. The method of claim 1, wherein the administration is parenteral.
7. The method of claim 1, wherein the adjuvant is alum.
8. A method of treating or preventing insulin dependent diabetes mellitus in a patient, the method comprising administering to the patient an immunologically effective amount of a pharmaceutical composition comprising an adjuvant and a cyclic immunogenic MHC polypeptide from a hypervariable region of a HLA-DQ molecule.
9. The method of claim 8, wherein the hypervariable region is in the β chain of the HLA-DQ molecule.

10. The method of claim 8, wherein the cyclic immunogenic MHC polypeptide consists of about 20 to about 30 residues.

5 11. The method of claim 8, wherein the immunogenic MHC polypeptide comprises amino acid residues 57-78 of a protein encoded by DQB1*0302.

12. The method of claim 8, wherein the immunogenic MHC polypeptide has the amino acid sequence CAAEYWNSQKEVLERTRAELDTVC.

10 13. The method of claim 8, wherein the administration is parenteral.

14. The method of claim 8, wherein the adjuvant is alum.

15 15. The method of claim 8, wherein the immunogenic MHC polypeptide is administered prophylactically.

16. A pharmaceutical composition comprising an adjuvant and an isolated cyclic immunogenic MHC polypeptide from a hypervariable region of a HLA-DQ molecule.

20 17. The composition of claim 16, wherein the hypervariable region is in the β chain of the HLA-DQ molecule.

25 18. The composition of claim 16, wherein the cyclic immunogenic MHC polypeptide consists of about 20 to about 30 residues.

19. The composition of claim 16, wherein the immunogenic MHC polypeptide comprises amino acid residues 57-78 of a protein encoded by DQB1*0302.

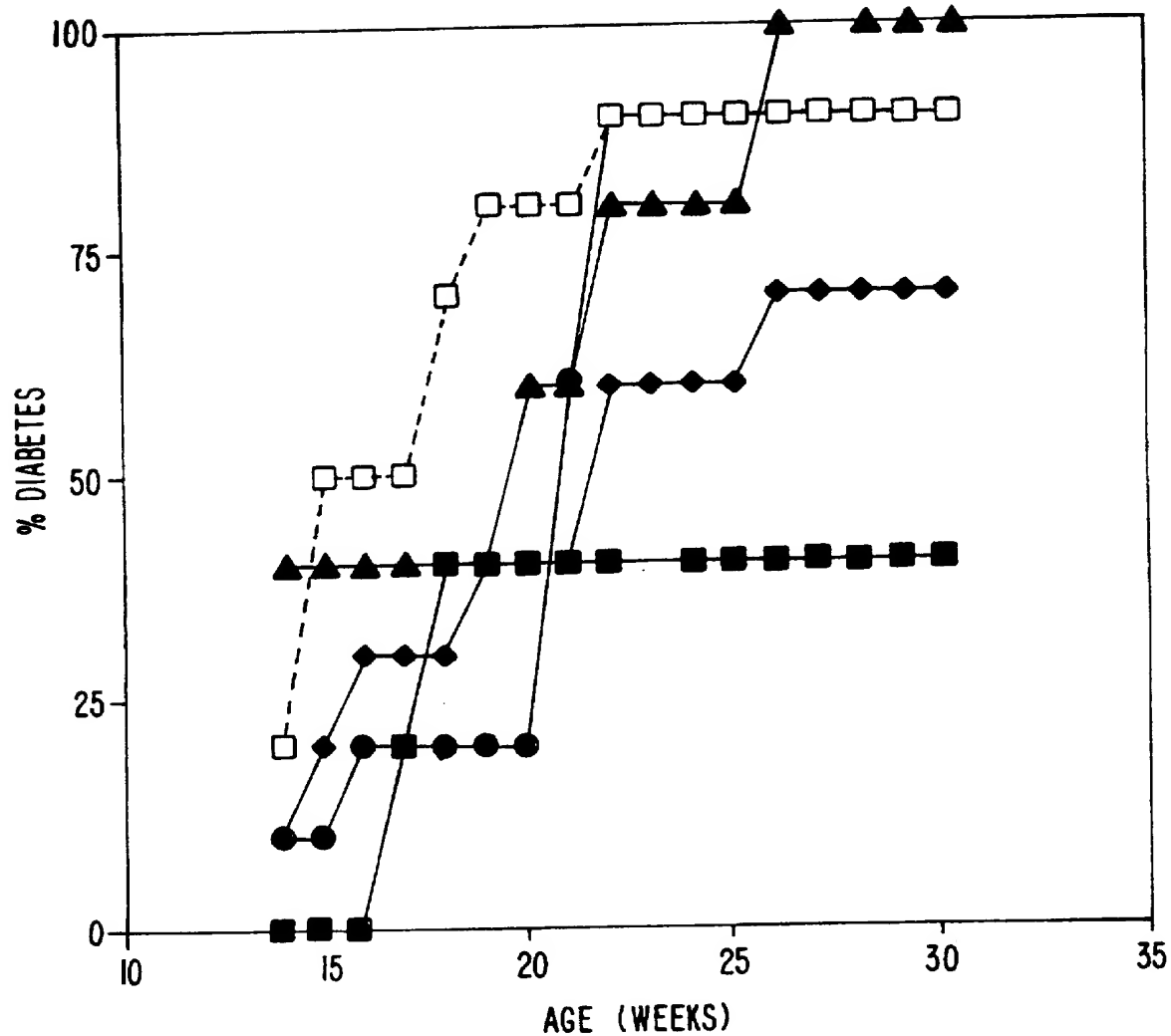
30 20. The composition of claim 16, wherein the immunogenic MHC polypeptide has the amino acid sequence CAAEYWNSQKEVLERTRAELDTVC.

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<u>HUMAN</u> DQ B1 0302 DR B 0401	A	E	Y	W	N	S	Q	K	E	V	L	E	R	T	R	A	E	L	D	T	V
	D	E	Y	W	N	S	Q	K	D	L	L	E	Q	T	R	A	A	V	D	T	V
<u>MOUSE</u> IA g7 IA S	S	E	Y	W	N	S	Q	*	*	Y	L	E	R	T	R	A	E	L	D	T	A
	D	E	Y	W	N	S	Q	*	*	Y	L	E	Q	T	R	A	E	L	D	T	V
<u>RAT</u> RT1 BB1 RT1 BBS	S	E	Y	W	N	S	Q	*	*	Y	L	E	Q	T	R	A	E	L	D	T	V
	D	E	Y	W	N	S	Q	*	*	Y	L	E	Q	T	R	A	Q	L	D	T	V

FIG. 1.

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- □ - ALUM (n=15)

- ▲ - 5µg (n=5)

- ◆ - 50µg (n=10)

- ● - 100µg (n=10)

- ■ - 200µg (n=7)

FIG. 2.

SUBSTITUTE SHEET (RULE 26)

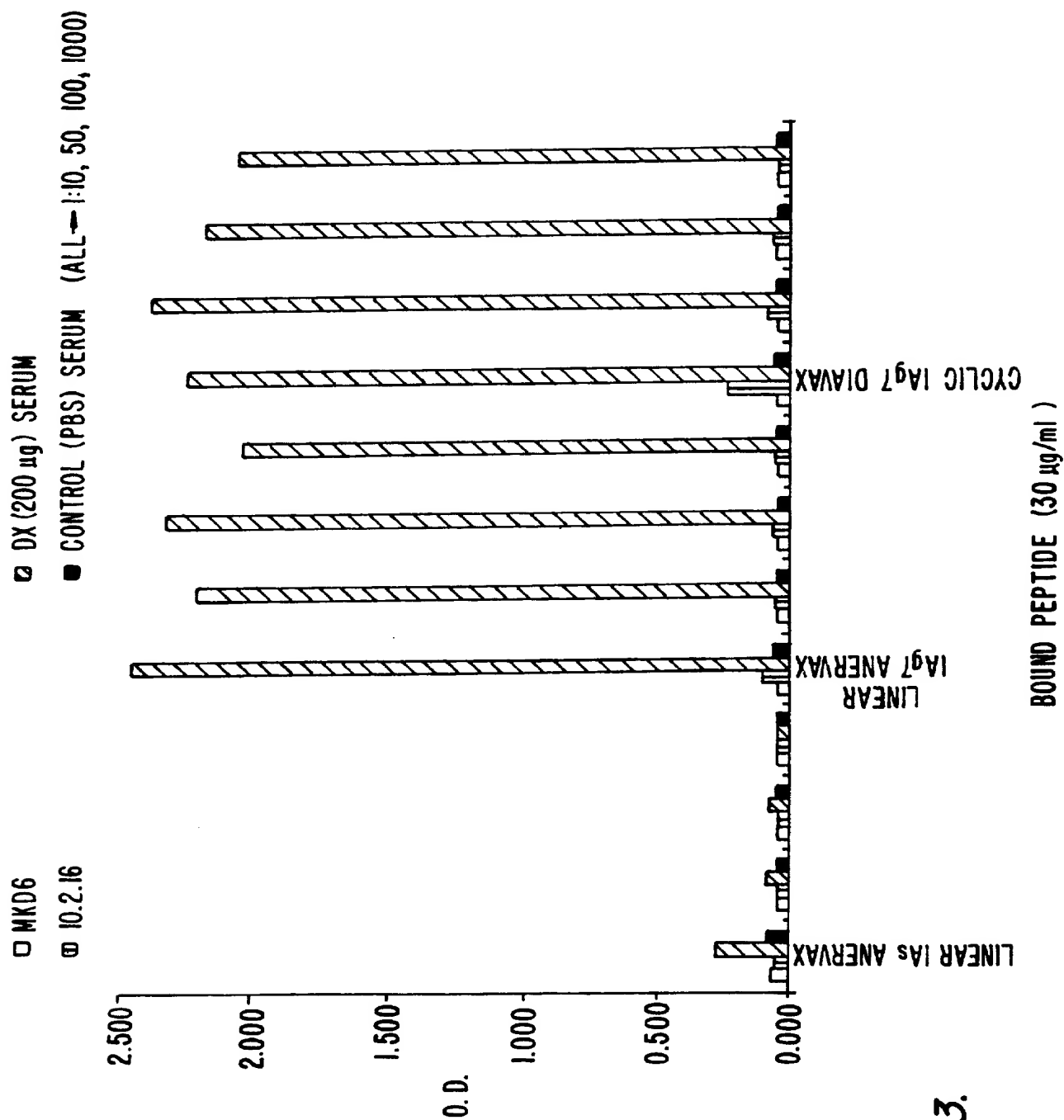


FIG. 3.

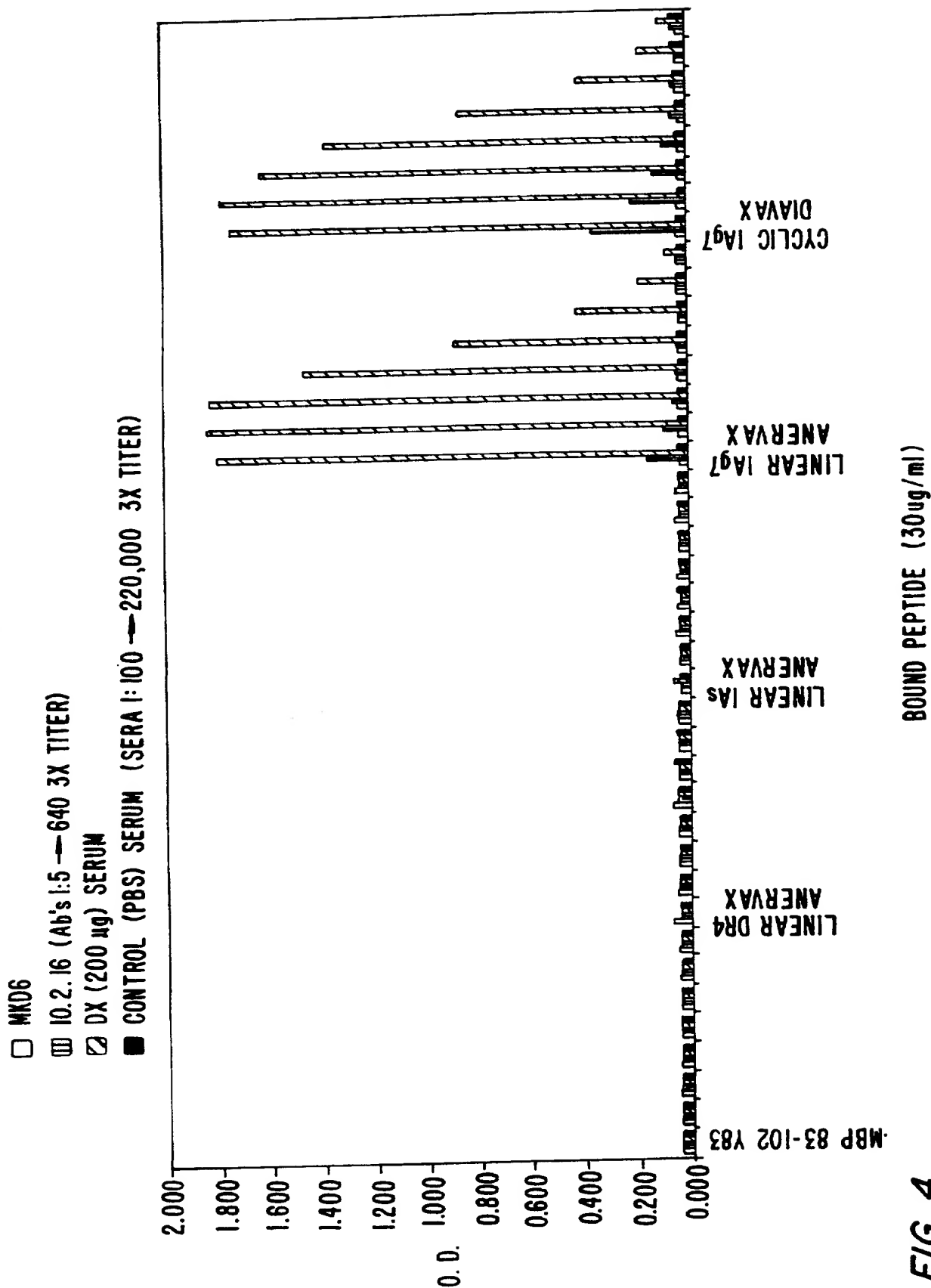


FIG. 4.

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TREATMENT				ANTIBODIES TO:							
AGE	WKS - BOOST	TREATMENT	DOSE	BLANK	MBP	I-As	DR4	LINEAR -Ag7	CYCLIC -IAg7	LINEAR-IAg7 56 (R->O)	CYCLIC-IAg7 56 (R->O)
3	4	DvX	100	0	0	0	0	8.3	8.3	33.33	0
3	6	DvX	100	0	0	0	0	23.1	23.1	23.1	23.1
3	4	ALUM		0	0	0	0	0	0	0	0
3	6	ALUM		0	0	0	0	0	0	0	0
4	4	DvX	100	0	0	0	0	54.5	54.5	54.5	54.5
4	6	DvX	100	0	0	0	0	41.6	41.6	41.6	41.6
4	4	DvX	50	0	0	0	0	0	0	0	0
4	6	DvX	50	0	0	0	0	0	0	0	0
4	4	ALUM		0	0	0	0	0	0	0	0
4	6	ALUM		0	0	0	0	0	0	0	0
5	4	DvX	100	0	0	0	0	50	50	50	50
5	6	DvX	100	0	0	0	0	63.6	63.6	63.6	63.6
5	4	ALUM		0	0	0	0	20	0	0	0
5	6	ALUM		0	0	0	0	14.2	14.2	14.2	14.2
6	4,10	DvX	100	0	0	0	0	60	60	60	60
6	4	DvX	100	0	0	0	0	60	60	60	60
6	6	DvX	100	0	0	0	0	33.3	33.3	33.3	33.3
6	4	DvX	50	0	0	0	0	0	0	0	0
6	6	DvX	50	0	0	0	0	0	0	0	0
6	4	ALUM		0	0	0	0	0	0	0	0
6	6	ALUM		0	0	0	0	0	0	0	0

FIG. 5.

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TIME TO SAC	CELLS	PRIMARY TREATMENT	SECONDARY TREATMENT							
			CON A	LPS	GAD 65 524 -543 NH2		MSA-OH	LINEAR I-Ag7	DiavaX	
			% OF MICE RESPONDING							
4 WEEKS	LYMPH NODE	ALUM	100	100	0		0		0	0
		PBS	100	100	0		8		0	0
		50µg DiavaX	100	100	0		0		0	0
		100µg DiavaX	100	100	0		0		0	0
		200µg DiavaX	100	100	0		0		0	0
	SPLENOCYTES	ALUM	100	100	0		0		0	0
		PBS	100	100	0		0		0	0
		50µg DiavaX	100	100	0		0		33	16
		100µg DiavaX	100	100	0		0		66	33
		200µg DiavaX	100	100	0		0		100	66
6 WEEKS	LYMPH NODE	ALUM	100	100	0		0		0	0
		PBS	100	100	0		0		0	0
		50µg DiavaX	100	100	0		0		0	0
		100µg DiavaX	100	100	0		0		0	0
		200µg DiavaX	100	100	0		0		0	0
	SPLENOCYTES	ALUM	100	100	0		0		0	0
		PBS	100	100	0		0		0	0
		50µg DiavaX	100	100	0		0		33	33
		100µg DiavaX	100	100	0		0		83	75
		200µg DiavaX	100	100	0		0		100	100

FIG. 6.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05461

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 33/06, 38/12, 38/16; C07K 14/74

US CL : 424/698; 514/9, 13; 530/317, 325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/698; 514/9, 13; 530/317, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN medline biosis scisearch embase lifesci, APS

search terms: MHC, HLA, cyclic peptide#, immun?, HLA DQ, diabetes

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,196,308 A (NEPOM et al) 23 March 1993, see entire document.	1-3, 6-10, 13-18
Y	LIU Z. et al. T cell recognition of allopeptides in context of syngeneic MHC. J. Immunol. 01 January 1992. Vol 148, No. 1, pages 35-40, See entire document, especially abstract.	1-3, 6-10, 13-18
Y	VITALE M. et al. Production and characterization of murine monoclonal antibodies recognizing HLA-DQ polymorphisms obtained by immunizing mice with transfected L cells. Hum. Immunol. June 1992, Vol. 34, No. 2, pages 126-134, see entire document.	1-3, 6-10, 13-18



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 MAY 1997

Date of mailing of the international search report

14 AUG 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05461

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRADLEY D.S. et al. Anti-HLA-DQ antibody therapy reduces the incidence and severity of collagen-induced arthritis in HLA-DQw8 transgenic mice. Arthritis and Rheumatology. September 1995. Vol 38, No. 9(supplement), page s371, particularly abstract 1306.	1-3, 6-10, 13-18
Y	LIPTON R.B. et al. Autoimmunity and genetics contribute to the risk of insulin-dependent diabetes mellitus in families: Islet cell antibodies and HLA DQ heterodimers. Amer. J. Epidemiol. 01 September 1992. Vol 136, No. 5, pages 503-512, see entire document.	8-10, 13-18